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Direct Mutagen Risk Assessment
The Development of Methods to Measure Immunologic and Genetic Responses to Mutagens

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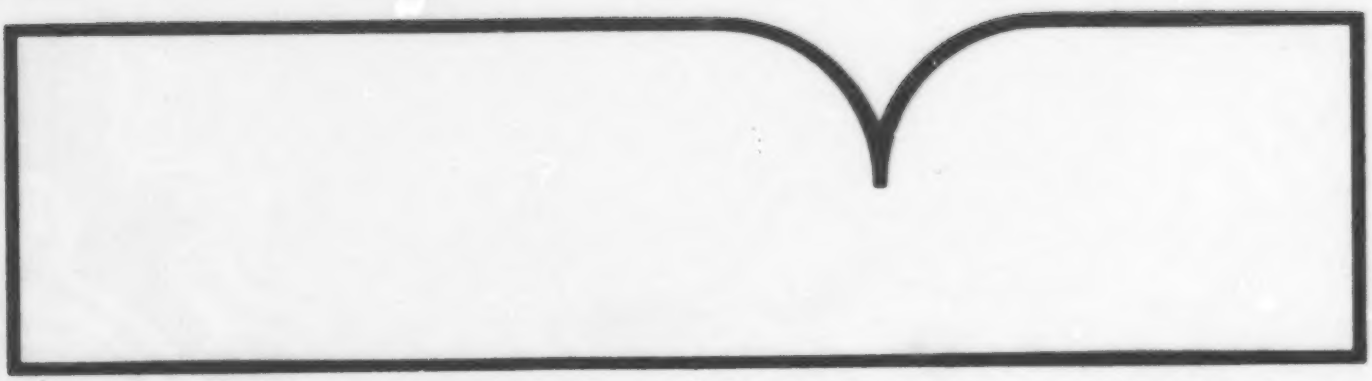

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DIRECT MUTAGEN RISK ASSESSMENT; THE DEVELOPMENT OF METHODS TO MEASURE IMMUNOLOGIC AND GENETIC RESPONSES TO MUTAGENS

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16. ABSTRACT

This review puts forward the concept of Direct Mutagen Risk Assessment through a discussion of the development of methods with which to measure immunologic and genetic responses to mutagens. Mutagenicity risk assessment, in this context, is the study of human somatic cell mutation occurring in vivo and is considered with attention to ideal criteria for detection of mutant cells. Immunotoxicity is addressed particularly in terms of the possible carcinogenic consequences of impaired cutaneous cell-mediated immunity.

A section of this reveiw reports the development and the exploitation of the Strauss-Albertini test for enumerating 6-thioquanine-resistant peripheral blood lymphocyte variants. Another section presents results from a system under development, designed to clone and quantitate mutant lymphocytes taken directly from the body. The Clonal Assay of Lymphocyte Mutagenesis (CALM) can confirm that variant cells are mutant and, potentially, could be used comparatively to measure genotoxicity in human or animal lymphocytes after in vivo or in vitro exposures to mutagens. The final section describes experiments to evaluate both immuno- and genotoxic effects of psoralen plus ultraviolet A (PUVA) including cancer-causing effects. In conclusion, the review emphasizes the need for an individualized approach to the evaluation of susceptability to mutagenic agents in terms of identifiable damage to human health.

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Accepting society's addiction to the products of industrial technology it is our responsibility to try to ascertain the adverse effects of chemical and physical agents. An escalating awareness of the potential and manifest severities of mutagen-related health effects confers a sense of urgency upon the problem of producing and utilizing relevant data for sound risk-versus-benefit predictions and decisions. The purpose of this disquisition is to review our efforts to develop methods with which to evaluate the *in vivo* effects of mutagen exposure in cells taken directly from the body.

The direct-testing approach offers several important advantages for evaluating the consequences of mutagen exposure in humans: (i) all relevant metabolic and pharmokinetic processes are intact and active; (ii) individuals (and families) may be assessed for differences in susceptibility to effects of various agents; (iii) spontaneous (background) mutation frequencies may be determined and monitored so that the consequences of exposure to complex mixtures of mutagens and other relevant environmental factors or to extraordinary exposures may be studied; and (iv) mutagenicity test results may be compared directly with those of conventional clinical studies of the same individuals and with epidemiological findings from their peers.

A primary goal of direct mutagenicity tests should be to detect and quantitate genotypic variance. The detection methods should be based upon an unambiguous phenotype at the single-cell level, so that mutant cells can be distinquished from normal cells. The specimens should be easily

obtainable tissues containing cells of polyclonal origin, taken directly from the body. Currently, the most promising approaches, though they remain in developmental stages, are those designed to detect mutations at specific gene loci. Among these are 2 monospecific antibody methods. One detects human red cells containing variant (sickle cell, HvS) hemoglobin (Stamatoyannopoulos et al., 1980) and the other detects an altered sperm-bearing antigen (lactodehydrogenase-X) which differs from the normal form by just one amino acid (Ansari et al., 1980). Severe deficiencies will exist in these new systems until processes underlying the development of variant cells can be more fully characterized (Malling, 1981). The third evolving approach in this category is the Strauss-Albertini test with which one can demonstrate that the drug resistant variant cells are indeed products of mutant genotypes (Strauss and Albertini, 1977, 1979).

This method has been formulated and modified with attention to ideal criteria for identifying cells as mutant on the basis of phenotype as follows:

1. occurs randomly and at low frequency;
2. remains stable in the absence of selection;
3. increases in frequency (and in some cases, in reversion rate) after exposure to mutagens;
4. can be associated with an altered gene product (usually a protein);
5. is attributable to a specific chromosomal region and behaves in a Mendelian fashion;
6. allows viability;
7. can be isolated and identified unambiguously at the single-cell level;
8. exists in the haploid state, obviating dominance-resessive relationships (this would require an X- or Y- chromosomal location, although an autosomal locus will suffice for cells in the heterozygous condition prior to testing or having the appropriate small deletion on the homologous chromosome);
9. two possible causes of the altered phenotype, chromosome deletions and specific gene mutations, can be distinguished from each other;

10. is transferable as a marker to other cells;
11. is detectable in the presence of a vast majority of normal cells under selection and can be propagated for further characterization.

(Thompson and Baker, 1973; Clements, 1975; Chu and Powell, 1976).

The Strauss-Albertini test is the descendant of human cell in vitro test systems which rely upon the ability to select, quantify and isolate mutant drug-resistant cells occurring spontaneously or as a consequence of exposure to test mutagens for example: (DeMars, 1974; Thilly et al., 1976).

The marker phenotype most commonly used in these systems is purine-analogue resistance, conferred as a result of DNA damage to the X-chromosomal gene (Hpt locus) responsible for the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Virtually all cells from males afflicted with the germinally inherited X-linked recessive Lesch-Nyhan (LN) Syndrome are HGPRT deficient and therefore survive using the de novo pathway for purine-base processing in DNA synthesis, in the presence of the purine analogue 6-thioguanine (TG) at concentrations grossly cytotoxic to normal cells (Kelley, 1968; Demars, 1974).

By design, the Strauss-Albertini Test avoids many questions of metabolic realism and target relevancy to which the in vitro mutagen exposure systems are subject. Peripheral blood lymphocytes (PBLs) are easily sampled, receive exposure to conditions extant throughout the body, are easily triggered to DNA synthesis, have been well characterized as to their behavior under conditions of short-term

culture and various structural and functional properties. The basic approach, therefore, is to screen PBLs for the presence of LN-like cells. Variants which incorporate tritiated thymidine [^{3}H]-TdR *in vitro* in response to phytohemagglutinin (PHA) despite the presence of TG can be counted by light microscopy after autoradiography.

The method used to perform the earliest applications of the Strauss-Albertini Test can be found elsewhere (Strauss and Albertini, 1977, 1979; Strauss et. al., 1979, 1980a). A revised procedure (for details see Strauss, 1982; Albertini, 1982) is presented in Figure 1 and it takes into account the recent finding that PBLs cycling at the time of biopsy (or at least early in the culture period) can appear as TG-resistant (TG^r) variants. These cells, known as "phenocopies", may have resulted in overestimations of variant frequencies (vfs) determined for normal individuals (as indicated in figure 4) and those at risk. Preliminary results indicate that the phenocopies can be eliminated by freezing the PBL speciments before they are tested (Albertini, 1982).

The culture protocol was designed to evaluate the products of *in vivo* events, providing sufficient time for vigorous DNA synthesis while denying cell division *in vitro*. Nuclei were freed from PHA-agglutinated cells for enumeration and it was shown that all persist to be counted.

The selective concentration 2 x 10^{-4}M TG was chosen on the basis of comparative dose-response studies wherein nearly all PBLs from LN males were resistant [variant frequency (Vf)=7.5x10^{-1}] and PBLs from normal individuals were sensitive (Vf=9.0x10^{-5}). TG^r PBLS from LN heterozygotes were observed at an average Vf of 2.0x10^{-3}. The Strauss-Albertini test was presented as a means to diagnose LN mutation carrier status in females presumed to be at risk. (Strauss et al., 1980a). The absolute sensitivity of the assay was estimated to be at least 10^{-5} by evaluating cultures containing artificial mixtures of minority LN PBLs in majority populations of PBLs from a normal individual. These results suggested that efficiency of recovery was not affected by any in vitro process of selection against the mutant phenotype.

In a series of 98 experiments TG^r PBL frequencies were determined in 63 healthy, non-LN individuals ranging in age from 11 to 75 years. The median Vf was 1.0x10^{-4} (the mean was 1.3x10^{-4}, and the 10th and 90th percentiles were 5x10^{-5} and 1.5x10^{-4} respectively). No correlation between age of donor and Vf was found. We suggested that though many of the pertinent T-lymphocytes are long-lived, failure of TG^r PBLs to accumulate with time may be explained, in part, by in vivo selection against the well-differentiated cell. Evans and Vijayalaxmi, (1981) have reported an age-dependent increase in 8-azaquanine resistant lymphocytes which they conclude may represent single Xq deletions. This issue obviously requires further inquiry. In the case of the LN heterozygotes, the finding of mosaicism at less than 50% is assumed to result from negative selective pressure against the mutants both at the stem-cell level and at the level of PBLs at advanced stages of maturity.

We conducted a cross sectional study of a group of cancer patients heterogeneous in terms of tumor type and treatment regimen. Results from patients with haematological malignancies are excluded from data presented here. They received a variety of combination cytotoxic therapies including antineoplastic drugs and x-irradiation over widely variable time courses. PBLs from twenty normal controls and 11 treated cancer patients were tested at $2x10^{-4}$M TG, while cells from 24 controls and 42 patients were studied at $2x10^{-3}$M TG. As Figure 2 shows, the average Vf at the higher TG concentration for normal controls was $8.7x10^{-5}$, and at lower TG concentration increased slightly to $1.3x10^{-4}$. Values from the single control tested 20 times at the higher TG and 5 times at the lower TG concentration, except for the outlying low frequencies from two test, showed variability and a range similar to those for the control group. The distributions of Vfs for treated cancer patients clearly were different from those of the normal controls. Eight of 11 patients tested at $2x10^{-4}$ TG and 26 of 42 patients tested at $2x10^{-3}$M TG showed Vfs higher than the highest seen in a normal person tested at the same TG concentrations.

With reference to the data from this cancer patient study the relationship between labelling indices of non-inhibited PHA-stimulated cultures and Vfs was studied. It was concluded that elevated Vfs was studied. It was concluded that elevated Vfs were not necessarily correlated with labelling indices lower than in controls. In fact, PBLs from 49 of 53 cancer patients responded normally to PHA within the

period of assay. Presently it is not known to what extent phenocopies contributed to these data, however owing to the secondary leukopoietic effects of cytotoxic drugs it would not be surprising to find enhanced frequencies of cycling cells in these people.

Preliminary studies* (Strauss and Albertini, unpublished results) indicated that untreated breast cancer patients, prior to any treatment, show slightly elevated TG PBLs, which appeared to become greater following surgery and adjuvant chemotherapy. Indeed one might expect in vivo responses to antigens, be they associated with infectious agents, grafts or tumors, to produce increases in numbers of cells cycling in the peripheral circulation. Cycling cells might well be found to be useful indicators of alterations in test cell subpopulations responding to conditions extant in the body at any given time.

A longitudinal approach was adopted whereby each individual is monitored and assessed in terms of personal baseline (pre-exposure) values. This approach was used with some success in a variety of human, canine and rodent studies of Vf discussed elsewhere (Strauss, 1982b, 1982c). Efforts to assess some of the effects of PUVA (psoralen and near ultraviolet light) exposure using this approach are described below.

Though further longitudinal evaluations are required, it appears that the results of Strauss-Albertini test reflect the operation of a brief latency and patency period. Evidently phenotypic expression of TG^r PBLs is complete within several days following mutagenic exposures.

The length of this period (and the magnitude of genotoxic damage observed) probably depends upon the extent of specific and non-specific killing of PBLs, as well as upon associated states of lymphopoiesis. The persistance of the induced variants (usually numbering days or weeks) must also depend upon these factors plus the operations of negative selection mechanisms.

Further to characterize TG^r PBLs as to their mutant nature, studies were undertaken to measure growth of thes cells in vivo. The presence of TG^r PBLs in humans is, for the most part, unimportant to health except in LN individuals and in those administered purine analogues therapeutically. Renal graft recipients were evaluated prospectively to monitor the efficacy of azathioprine as an immunosuppressant for transplantation. Vfs increased considerably as many as 10^{-2} from normal levels in all 20 patients studied, returning to only moderately increased levels in recipients of well-matched kidneys from living-related donors; on the other hand, Vfs continued to climb in those given poorly matched grafts which, ultimately, were rejected (Strauss, 1982b). Again, the degree to which phenocopies may contribute in this situation remains to be determined. There is, however, little doubt that these TG^r cells were generated through a process of antigen-induced proliferation in the face of selection favoring the variant phenotype and indeed these may mediate rejection. In this context it is interesting to consider that in vivo phenocopies may provide a basis for the development of tumors and other somatic cell degenerative proliferative abnormalities.

The growth of TG^r PBLs also was measured *in vitro* through the use of two methods for producing long-term cell lines. PBLs were taken from normal donors and from a renal transplant patient having a Vf of nearly 10^{-2}, and cultures were infected with Epstein-Barr virus B95-8 in the presence and absence of TG. Though PBLs from each donor transformed and grew well in the absence of TG, only the PBLs from the transplant patient developed into an established B-cell line in the presence of TG (Strauss and Albertini, 1979; Albertini, 1979). In the second case similar results were obtained using T-cell growth factor (TCGF) to cause the propagation of a TG^r T-cell line (Lane, Strauss and Albertini, unpublished results). The latter study inspired the development of the clonal assay of lymphocyte mutagenesis (CALM) to isolate, characterize according to the criteria defining somatic cell mutants and enumerate the TG^r PBLs (Strauss, 1982b, see 1982d for complete details).

TCGF is a general term for a class of naturally occurring lymphokines, individually identified as Interleukins. TCGF-containing supernatants from PHA-stimulated PBLs can be used to establish and support the continuous T lymphocytes (CTLs) from blood, skin, lymph nodes, spleen, bone marrow and other tissues. The CTLs are not virus-transformed and maintain their morphological and functional integrity (Ruscetti et al., 1977; Gillis et al., 1978). TCGF can be purchased commercially or produced in large volumes in the laboratory from cultures of PHA-stimulated leukopheresed PBLs; its quality easily can be assessed by measuring its

influence on the growth of CTLs. CTL densities routinely were maintained between 0.5 to 1.0×10^6/cc by viability counting and refeeding with TCGF-containing medium, T-cell growth medium (TCGM) each two to three days.

Mesurements were performed to compare proliferative responses of CTLs in TCGM from two normals and two LN individuals in the presence of TG at various concentrations. An optimal selective concentration of TG, 2×10^{-5}M, was determined from the kill curves. In one set of experiments CTLS from normals at densities of 10^5/culture containing TG were completely eliminated by the eighth day of culture, whereas those from LNs were unaffected. To test the persistence of the TG^r phenotype in CTLs developed from a normal individual in the absence of selection, one set of 10^8 CTLs was challenged with TG. Survivors were raised under these selective conditions for ten days. By this time levels of about 10^6 cells were reached and the TG^r CTLs were placed in non-selective medium and cultivated for another ten days. At this stage, 0.5×10^6 TG^r CTLS were planted in selective medium and compared with the non-selected counterparts. The TG^r CTLS were unaffected, the others were killed. The results suggest that if selection against LN-like CTLs from normal individuals indeed occurs <u>in vitro</u> it is far from complete. Figure 3 depicts the procedure used in the early development of CALM. Lymphocytes were diluted in TCGM containing 0.05% x-irradiated sheep red blood cells, which served as filler/feeders; cells were then plated in paired 96-well microtiter plates at 10^5 cells per well in the presence of TG and at 1 cell per well in the absence of TG. Spent media were replaced with fresh media (at

5 and 9 days) and ^{3}H TdR label added on day 9. On day 10, activity per well was assessed sequentially by three comparable methods: colorimetry, light microscopy, and liquid scintillation spectrophotometry. By scoring wells positive or negative on the basis of these assays it was possible to calculate cloning efficiency (CE) assuming an ideal Poisson distribution. For each 96- well plate the number of cells per well resulting in the percent positive wells was divided by the number of cells per well plated. In this way, for each pair of plates, CEs were determined for cells TG-exposed and cells not TG-exposed such that the ratio $CE_{TCGM,TG}/CE_{TCGM}$ approximated in vivo (or for CTLs, in vitro) TG^{r}PBL mutant frequencies.

Cultures containing serial dilutions of minority PBLs from a LN boy were added to a majority of PBLs from a normal individual before plating, culture, and analysis as described. Expected total mutant frequencies (Mfs) were determined by summing the observed (Mf) for the normal individual and the expected Mf of LN PBLs (based on the known CE for these cells grown seperately). By this method efficiency of recovery (EoR) was judged to be rather high with a test sensitivity of at least $5x10^{-6}$. A second set of artificial mixture experiments was performed using minority TG^{R} and majority TG^{S} CTLs developed from the PBLs of one normal individual as described earlier. The EoR values again were consistant with the conclusion that in vitro selection against rare TG^{R} lymphocytes probably does not interfere with the performance of this assay. The results of this experiment suggest that cell-mediated cytolysis among alloreactive immunocytes was not problematic in the first EoR experiment (Strauss, 1982).

The Strauss-Albertini test and CALM were performed in parallel directly to compare *in vivo* and *in vitro* TG^r lymphocyte frequencies among fresh and cryopreserved PBLs and CTLs. Cells from two LNs were tested once, and cells from two normals were tested on four seperate occasions; Figure 4 provides the results of this exercise. LNs, as expected, produced Vfs and Mfs near unity in every case. Though they are few, the data seem to support the possibility that cryopreservation eliminates phenocopies from the assay. As this figure also shows, CALM Mfs, for CTLS and PBLs from the normal individuals are similar to each other and to Vfs from frozen PBLs (Strauss, 1982).

Though results are preliminary and the assay is still under development, it seems safe to suggest that CALM, as described here, (Strauss, 1982d) and versions now being developed in other laboratories (Albertini et al., 1982; Morely, et al, 1983) may aid in verification of the Strauss-Albertini Test and possibly may serve as a mutagenicity test system unto itself.

CALM is being adapted to the evaluation of genotoxic damage after *in vitro* exposures to various agents (Figure 5). Individual susceptibility to mutagens and the relevancy of detectable long-term and short-term health risks could then be assessed. In addition, it would be reasonable and valuable to use CALM in mice, both *in vivo* and *in vitro*. The response of target lymphocytes from diverse tissues

to a variety of agents could be assayed in mice (and in man to a lesser degree). Such studies would help to develop the means to extrapolate realistically the results from experiments with rodents to man (and back).

Further to answer critical questions concerning the relevancy of mutagenicity test systems, it is necessary to develop mechanisms directly to correlate their results with associated human health effects. New approaches to they study of mutagen-related effects are needed so that the acute, chronic, or latent effects of high-dose-single or low-dose-chronic mutagen exposures can be recognized and quantified. The need to develop new approaches seems especially urgent now, because it is clear that a subtle short-term health effect, either by itself or by interacting with other health effects, can produce long-term ill-health consequences through disturbances in normal regulatory processes. To explore some of these interactions, studies of psoriasis patients and others receiving PUVA oral psoralen plus whole body ultraviolet A photochemotherapy) treatments were conducted.

Psoralens are known to damage DNA following UVA irradiation by combining covalently with the pyrimidine bases of nucleic acids which then act as bivalent reagents capable of cross-linking opposite strands of the double helix. PUVA-generated lesions have been detected in a variety of test systems using bacteria, cultured mammalian cells, and human peripheral lymphocytes both <u>in vitro</u> and <u>in vivo</u> (Bridges, 1978). PUVA treatment protocols are designed to clear the symptoms of generalized psoriasis, mycosis fungoides, urticaria pigmentosa

and vitiligo by producing regulated phototoxicity in the skin (Parrish et al., 1974). Treatments are administered two or three times per week. The actual effective dose of PUVA depends on the interaction of several factors: intensity and duration of UVA exposure, level of active psoralen in the skin at the time of irradiation, and relative quality of protective pigmentation in the skin. The last parameter is described by an index of skin type from I to V in order of increasing ability to produce protective pigmentation in response to light exposure.

PUVA-treated individuals were evaluated using the Strauss-Albertini test. The results off this cross-sectional study are summarized in Figure 6 (for details see Strauss et al., 1979 and Strauss, 1982c). Although the concurrent control group was small, variant frequency values fit nicely into the distribution range reported from the standard reference group (mean given here as of sixty-three normal indivuduals). After testing the two groups of psoriasis patients, the additional groups of vitiligo patients were tested. The PUVA-treated vitiligo patients received delivered doses similar to those of the PUVA-treated psoriatics. Vitiligo patients were tested because it was not possible to find untreated, severely diseased psoriatics. Thus in testing psoriatics, it was not possible to separate the effects of PUVA, conventional therapies (including combinations of UVB, coal tar, steriods and methotrexate), and possible disease-state effects on PBLs. Vitiligo patients presented a useful alternative, because untreated patients are available, and the test cell (PHA-responsive PBL, a T-cell) does not play a direct role in the pathogenesis of the disease.

A normal, healthy 28-year-old individual volunteered to receive intensive PUVA exposurees over a period of 3 months and to be tested longitudinally for TG^r PBL frequencies. Several tests were performed prior to treatment and the Vfs were within the normal range. A second normal, untreated individual concurrently tested produced normal Vfs during the entire period of the study. The PUVA-treated subject received the highest tolerable doses twice weekly and a response was evident within five or six days, when Vfs climbed out of the normal range. They fell back to near normal when the rate of treatment was dropped to once weekly. Soon after treatment was discontinued, the subject's variant frequencies returned to the normal range. If changes in Vfs are assumed to be due to changes in the DNA, these results would demonstrate that PUVA can cause mutations and, thus presumably, initiation of carcinogenesis in exposed tissues. The results demonstrate that the effects of PUVA definately extend to the capillary beds, with considerable peripheral blood exposure. It is reasonable to expect that the responses of skin cells in situ are similar to those of the PBLs (Strauss et al., 1979; Bridges et al., 1981; Strauss, 1982a, Strauss, 1982c).

PUVA exposure causes the development of cutaneous tumors in shaved and hairless mice (Roberts et al., 1979). In man the first evidence of a PUVA-related carcinogenic effect was the result of an ill-fated effort to induce protective melanogenesis in 4 xeroderma pigmentosum (XP) patients. XP an hereditary disease which, owing to a deficiency in repair

of U.V.-induced damage is characterized by enhanced sensitivity to the carcinogenic effects of sun exposure. The PUVA-treated XP patients all developed multiple cutaneous neoplasms within months of exposure and one also presented with lymphatic leukemia. Based on this information, one might expect to find similar tumors in PUVA-treated psoriatics, who should exhibit normal repair capacities, following initiation of damage, but only after the theoretical lag period of several decades. Several unexpected phenomena were observed: (1) An increased incidence of skin cancer has been reported within 2 years of starting PUVA treatment; (2) Excess tumors were mainly of squamous (not basal) cell origin, and (3) these developed only in patients in whom premalignant foci might be expected due to DNA damage from agents which had produced skin cancer prior to PUVA or from previous therapeutic exposures to x-rays; arsenicals or combination topical coal tar and tar U.V. (Stern et al., 1979, 1984)

Considering these observations together with evidence that squamous cell carcinomas are subject to immune controls (Walder et al., 1971, Kinlin et al., 1979) and considerable animal data (Morison, 1981; Lynch et al, 1981; Kripke et al., 1981) demonstrating that U.V. alone and PUVA cause neurological impairment, we (Bridges and Strauss, 1980) hypothesized that the carcinogenic action of PUVA might be functionally that of a pseudo-promoter (permissive carcinogen) rather than (or in additon to) that of an initiator. It seemed to us that if potentially malignant skin cells are subject to surveillance by the cutaneous immune system, then

impairment caused by PUVA might promote the growth of such clones. Ironically (and this is a matter of some controversy) the aetiology of psoriosis principally may involve an element of autoimmunity which can be controlled by immunosuppressive agents (Strauss, 1982).

An incidental observation during the period of intensive PUVA exposure of the volunteer subject discussed above provided new insights into both the beneficial and adverse effects (short-and long-term) of PUVA therapy. The subject was involved coincidently with immunological testing for clinical purposes not related to PUVA. Prior to PUVA exposures he had been sensitized against the skin-test reagent 2,4-Dinitrochlorobenzene (DNCB) and repeatly was found to be a high-normal responder. However, during the entire duration of PUVA treatment he was unresponsive to DNCB. This was discovered serendipitously, and no careful analysis of the phenomenon was attempted at the time. When the treatment period ended, normal reactivity to DNCB returned. The DNCB test measures cell-mediated immunocompetency by evaluating delayed cellular hypersensitivity (DCH) in the skin.

A cross-sectional study of DCH was performed in psoriatics, undergoing varying degrees of PUVA therapy. The results are summarized in Figure 7 (for details including testing and grading methods, refer to Strauss et al., 1980b). Fifty-five (roughly 54%) of the PUVA-treated psoriatics had sub-normal responses to DNCB sensitization. Under ordinary PUVA-treatment conditions there is a striking correlation between skin type and DCH grade. For each skin type

the average frequency of sub-normal responses was as follows: type I, 74%; type II, 73%; type III, 65%; type IV, 24%, type V, 0. X^2 analysis of the data from Figure 7 indicates that for all PUVA-treated groups (spanning 1/2-log ranges from 0.07 to 7.0 $J/cm^2/day$), observed DCH grades were dependent upon skin type, and that abnormal results are significant ($p<0.005$). Though they demonstrate that PUVA impairs cutaneous DCH, these studies did not differentiate between failure to respond to sensitizaiton and diminished challenge-response ability.

A second, longitudinal study, was designed to examine this question further (Strauss, G.H.S., D. Phil. Thesis, Sussex U., 1982). In summary, 20 psoriasis patients were DNCB sensitized and challenge-tested to determine individual optimal allergic responser prior to any PUVA exposure and at 6 wk intervals thereafter for a period of at least one year. Tests determined local DCH reactions both at skin sites exposed to and masked from UVA exposure. A normal, healthy control, who was not PUVA-exposed, was challenged at 8 wk intervals for a period of 2 years and consistently responded within normal limits. Challenge tests on pre-treatment psoriasis patients revealed DCH response distribution matching or slightly exceeding expectations for normal.

Briefly, the results of this study suggest a direct dose-response relationship between PUVA therapy and immune depression under ordinary treatment conditions and, in some cases, considerable impairment of cutaneous DCH. Sites exposed to UVA are more susceptible to DCH impairment

than are non-exposed sites. In general, at moderate doses in indivuals with low UVA tolerance and at high doses in those of all skin types, impairment of DCH at exposed sites is evident and can be dramatic. In fact, at exposed sites, five of thirteen low skin type patients became anergic to a DNCB dose (48.0 mg) sufficient to sensitize normal individuals.

Among individuals of high skin type, three of seven developed allergy to 48.0 mg DNCB following PUVA doses far greater than those required to cause the same effect in the low skin type group. Further, among low skin type individuals under the influence of the most intensive treatments, two individuals were rendered completely anergic to DNCB at both exposed and unexposed sites. In contrast, none of the higher skin type group became anergic to DNCB at unexposed sites under any of the treatment conditions. Our findings indicate that PUVA alters both local and systemic immune responsiveness following a short latency period of a day or two, whereas the effect on DCH persists one to two weeks.

Studies of Guinea-pigs using simulated PUVA treatment have provided results similiar to those described here (Morison et al., 1979; Vella-Briffa et al.; 1981; Morison et al., 1981). When UV-induced skin tumors carrying strong tumor-associated antigens were transplanted to syngeneic mice that had received PUVA, the grafts were accepted; non-treated hosts rejected the tumors immediately (Roberts et al., 1979). This and other animal studies have suggested that alterations in regulatory mechanisms within the immune system (e.g. supperessor T-cell induction) may be responsible for decreased responsiveness to antigenic stimuli

(but see Kripke et al., 1981, for a different interpretation of the results). Epidermal Langerhans cells ordinarily have antigen-presenting functions necessary for contact hypersensitivity to allergens and have been found to be exquisitely sensitive to PUVA. Their disappearance from the skin in mice has rendered the skin insensitive to topical immunizing doses of DNCB; further, it is probable that UV-damage antigen-presenting cells move to the spleen and become nonfunctional splenic adherent cells (Lynch et al., 1981). In addition to its action on afferent mechanisms required for contact-DCH, PUVA may alter efferent mechanisims by inducing specific suppressor lymphocytes, which limit the number of effector cells available to respond to challenge (Roberts et al., 1979; Claman and Miller, 1980; Letvin et al., 1980).

CONCULSION

In conclusion an anecdotal observation is offered to emphasize the central theme of this review. (Similar findings have been documented more completely; see (O'Dell et al., 1980).

The subject, a normal, healthy, middle-aged man, received moderate sunburn over much of his surface (skin type I) while windsurfing during a unusually pleasant English summer's day. The individual, who in the past had responded normally to sensitization and challenge, was now challenge-tested with three doses of DNCB on each arm. The DNCB was applied to the sunburned side of his right arm and to an area that clearly was not sunburned on his left arm.

Within the test period, normal DCH responses were observed on the left arm but not on the right arm. Evidently, sunlight exposure was sufficient to cause local cutaneous anergy. Whether such immunological tolerance in the presence of foreign entities (including infectious disease agents and tumor cells) promotes disease remains a matter for speculation. If, for example, the hapten DNCB goes unrecognized, so too might the transplantation antigens of squamous carcinomata. It is very likely, though, that mutagens cause both cytotoxic responses and mutations. These effects may interact over widely ranging time courses to cause disturbances in various normal regulatory processes. A major objective for genetic toxicology in the future should be to identify all relevant short-and long-term health risks from mutagen exposure.

In the near future, direct testing methods should be developed to the point where they can be used to predict manifest disease. Predictions should be based upon a developing understanding of the interactions between exposure and susceptibility. Direct testing methods should be developed so that:

1. results from in vitro tests can be compared with the results from direct tests;
2. results from mutagenicity tests on non-humans can be compared with direct-test results;
3. results from direct mutagenicity tests on somatic and germ cells can be compared;
4. relevant short-term consequences of mutagen exposures can be recognized and quantified; and
5. individual and population susceptibility to mutagens can be predicted quantitatively.

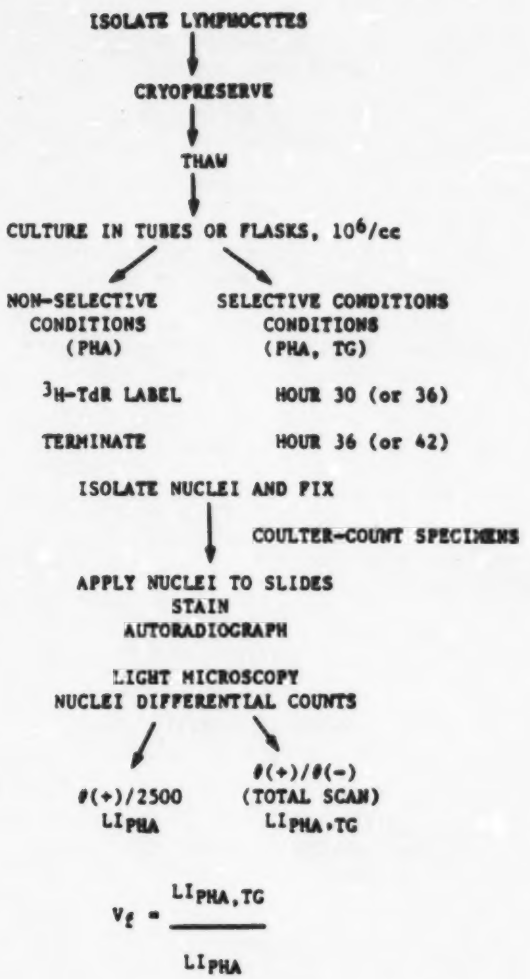

FIGURE 1. The Strauss-Albertini Test procedure.

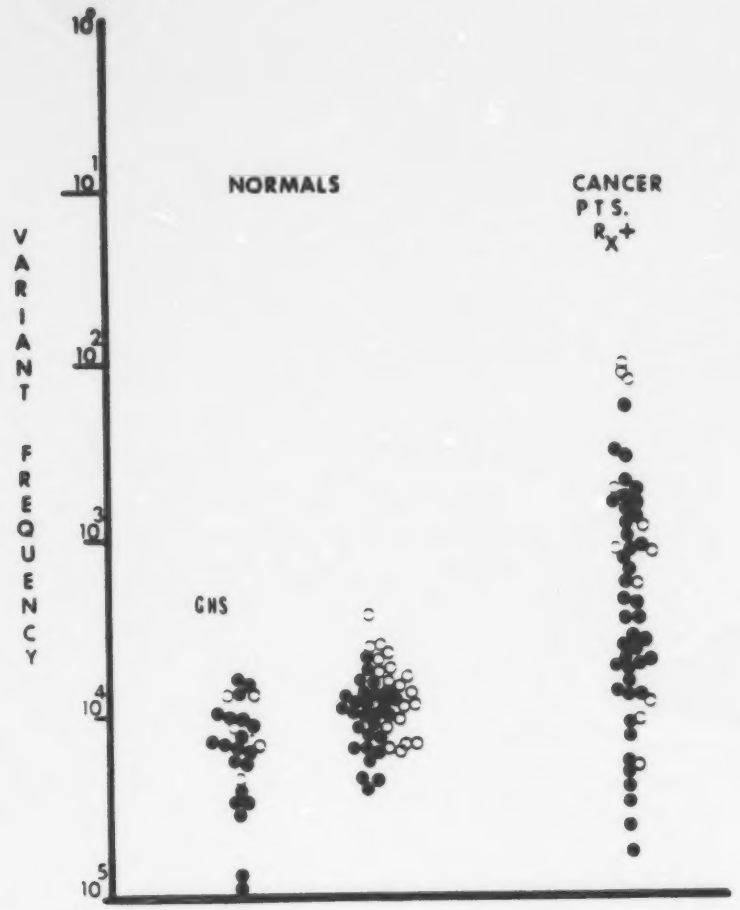

FIGURE 2. Frequency of TG^r PBLs in a heterogeneous group of cancer patients (right column), a group of normal controls (center column), and a single healthy individual (left column), determined at 2×10^{-4} M TG ( ) and 2×10^{-3} M TG ( ). Each point represents the results from one or more studies on an individual.

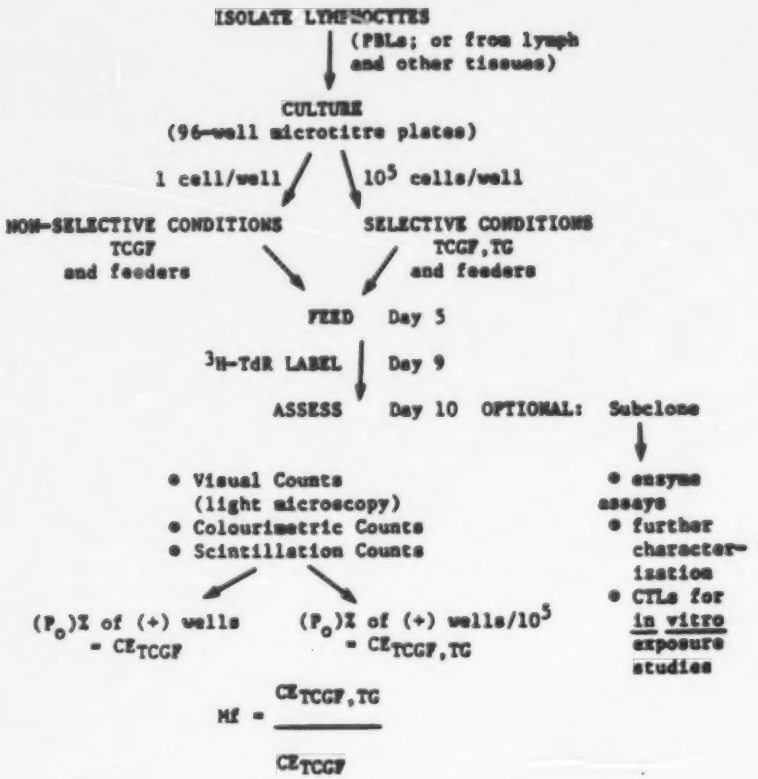

FIGURE 3. Clonal Assay of Lymphocyte Mutagenesis (CALM) procedure.

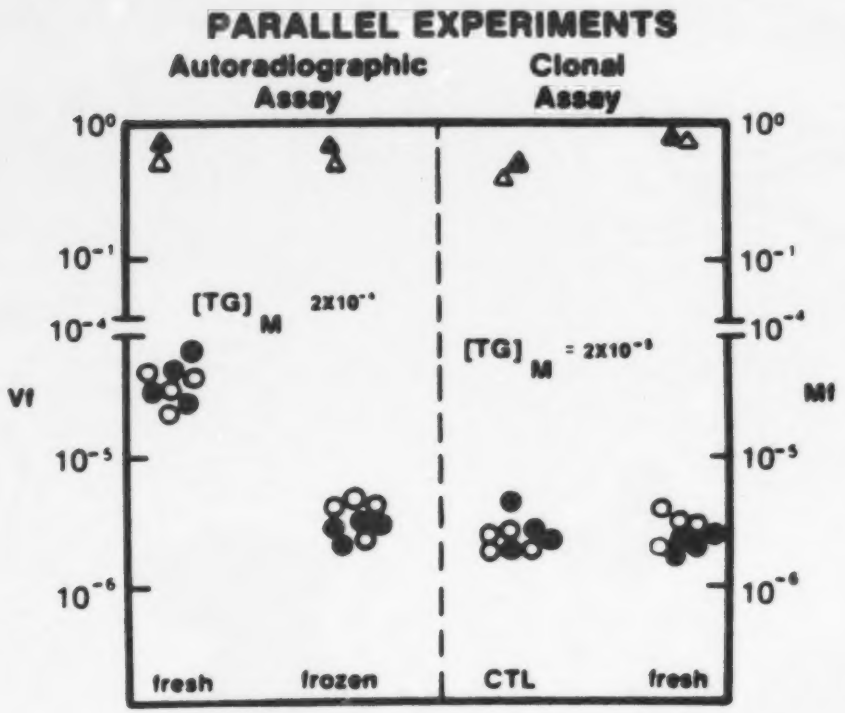

FIGURE 4. Scattergram of variant and mutation frequencies of PBLs from one parallel test of each of two L-N individuals (Δ, ▲) and from four separate parallel tests of two normal individuals (○, ●).

FIGURE 5. A possible procedure for the adaption of CALM to evaluation of genotoxic damage after in vitro exposurees to an agent.

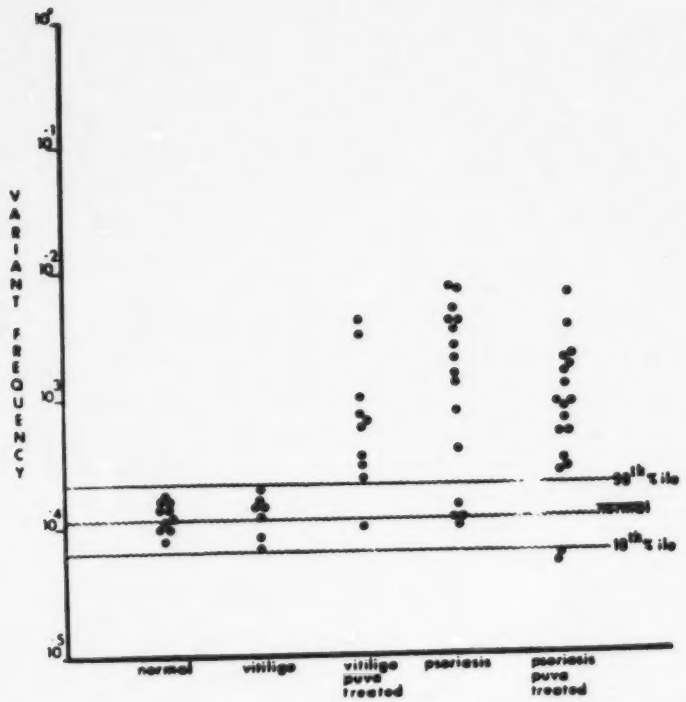

FIGURE 6. Frequency of TG^r PBLs as a function of treatment group; each point represents one test on one individual. The wavy line is the median variant frequency of 63 normal individuals.

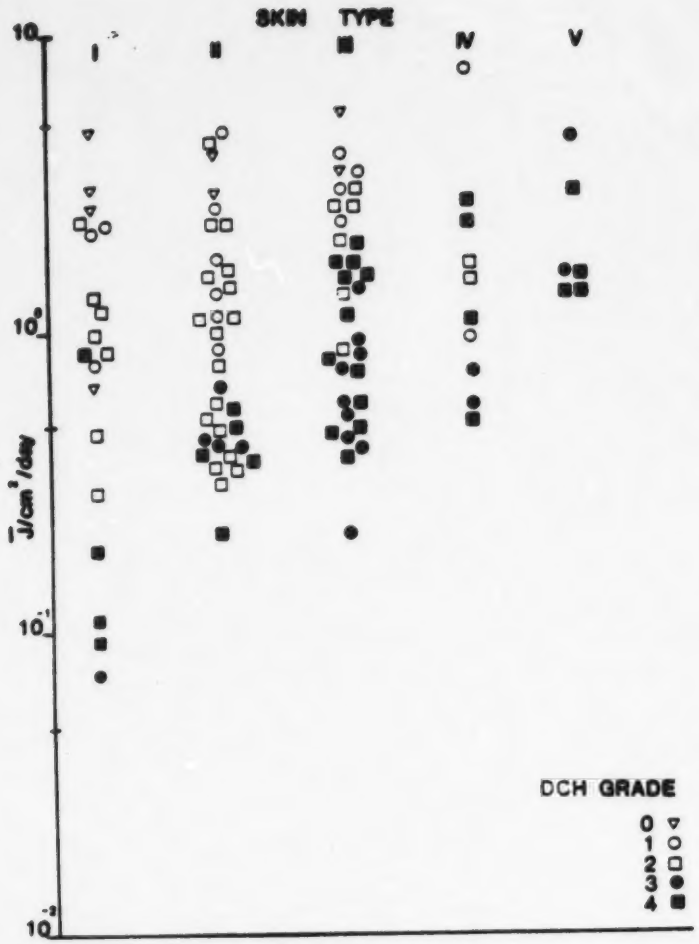

FIGURE 7. The interaction of a range of delayed cellular hypersensitivity (DCH) reactions (0-4), skin type (I-V), and dose rate ($J/cm^2/day$) in PUVA-treated psoriasis patients.

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